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RADIOACTIVE SODIUM PERMEABILITY AND EXCHANGE IN FROG EGGS

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Statement of the Problem

The transfer of ions in both directions across the cell membrane is a matter of major biological importance. While problems involving permeability have led to a vast and prolix literature, only meager attention has centered on the specific distribution of electrolytes within single cells. The limitations of earlier techniques were emphasized by Hastings (1941) in his Harvey lecture on the tissues and body fluids, in which he early recognized the value of radioactive isotopes. The subject of permeability has been recently reviewed by S. C. Brooks (1945). Our interest has centered in the dynamic equilibria of a protoplasmic system, their control by diffusion and the blocking of internal exchange. Use of radioactive tracer techniques has given new precision to the qualitative and quantitative study of small ionic transfers within cellular dimensions. Accordingly, in this paper are given results of an investigation undertaken to determine how much and how fast traced sodium (containing Na^{24}) exchanges with normal sodium (Na^{23}) in a vertebrate egg.

Materials and Methods

Ovarian "winter" eggs of the frog, *Rana pipiens,* were chosen because they are single cells, spherical and of large size. Throughout the fall and winter quarters of the year, they are readily available in quantity (without hormone stimulation). In addition to the vitelline membrane, ovarian eggs possess two thin epithelial layers which could be removed successively by fine-pointed forceps. However, in most of the experiments, these membranes were left intact, because of the possibility of injury to the cell surface. Amphibian eggs are ideal for volume measurements. One unique advantage is that they possess an extremely large nucleus, or germinal vesicle, approximately 0.5 mm. in diameter, which may be isolated easily under a dissecting microscope in a Ca-free medium (Duryee, 1937).

Eggs were isolated individually in standard Ringer solution: NaCl, 0.66 g.; KCl, 0.014 g.; $CaCl_2$, 0.012 g.; distilled H_2O, 100 ml. All solutions were buffered to pH 7.6 with $NaHCO_3$. As described below, Na^{24} was incorporated in experimental solutions as NaCl.

Active healthy frogs were selected. After ovaries of pithed animals were removed as bloodlessly as possible, fresh Ringer solution was poured over them before transfer to small beakers. A small lobe of an ovary was next cut off with iridectomy scissors taking care not to injure the cells. Groups of 20–30 eggs were next transferred to Syracuse dishes where individual eggs were cut apart. All small yellow or transparent eggs were removed and the ovarian wall was trimmed off flush with the theca membranes of each full-size egg. In this way, using a dissecting microscope to insure precise excision, it was possible to obtain from 40 to 60 separate eggs per hour. Average volume was 2.86 mm^3, based on an average diameter of 1.76 mm. An important step was the reexamination of individual eggs to reject those which might have been nicked or crushed. Aspherical eggs were also discarded along with any less than 1.65 mm. in diameter. Following these procedures, a final screening for uniformity of diameter was made. This consisted of shaking approximately 100 eggs in a flat bottom shallow dish to obtain a single layer with close packing. Any cell not obviously tangent to its six neighbors was discarded.

Eggs were conveniently handled individually with a pipette having an internal diameter just larger than that of the cell itself. In a few experiments where short exposures involving minimum transfer of external radioactive material was essential, each egg was advantageously picked up by the short flap of theca membrane with sharp forceps.

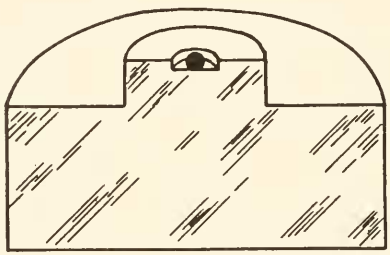

FIGURE 1. Sectional view of plexiglas cup for measuring radioactivity in frog egg. Actual size.

For experiments on dead cells, a method of killing involving minimal change was sought. After rejecting reagents such as alcohol, acetic acid and KCN, we standardized on heating to 65° C. for ten minutes in normal Ringer.

Groups of 10–20 eggs were exposed in small beakers containing 20 ml. of Ringer. Temperature was maintained in water baths controlled to $\pm 0.5°$ C. The presence of water vapor surrounding the beakers kept down evaporation, which by weighing was found to be negligible over periods up to 24 hours.

Upon removing a cell from the radioactive Ringer, it was put through two washes of normal Ringer before transfer to the measuring cup. One or two eggs were first transferred in a vertically held pipette allowing them to sink to the meniscus. By merely touching the surface of the washing fluid, the eggs dropped

from the pipette, thus avoiding transfer of more than one or two cubic millimeters of previous fluid. Washing vials each held 7 ml. It was considered advisable to use a fresh pipette for each successive transfer. Since it was found that Na^{24} could be washed from the cell interiors, the process was reduced to a standard thirty seconds.

For measurement in the Geiger counter, eggs were transferred from the second washing vial to special plexiglas cups (see Fig. 1) again in a vertically held pipette. Not more than 5 mm^3 of the second wash fluid was thus transferred with the egg. Tests of this fluid showed negligible radioactivity.

Radioactive solutions

The radioactive Na^{24} was prepared in the cyclotron by bombardment of metallic sodium with 16 m.e.v. deuterons. The metal was dissolved in ethyl alcohol and converted into NaCl with 12 N hydrochloric acid. The mixture was then dried and ignited at red heat to produce a neutral salt free of organic material. Weighed amounts of the radioactive salt were converted into amphibian Ringer solution. To obtain a relation between quantity and radioactivity of Na^{24}, a known aliquot of the Ringer was evaporated to dryness and measured with a Geiger counter. A typical result was as follows: 1 ml. of amphibian Ringer was diluted to 500 ml. A volume of 0.039 ml. of this solution was dried and measured on the counter. Since 1 ml. of the Ringer contained 0.026 g. Na, the aliquot possessed $0.026 \times 1/500 \times 0.039 = 2.03 \times 10^{-7}$ g. Na. This sample gave 1600 counts/min. Thus 1000 counts/min. represented 1.27×10^{-7} g. Na.

For each solution made up with radioactive sodium, controls were made up with identical amounts of normal NaCl. The radioactivity of the solutions was such that the radiation level was less than an amount which would produce 1 r.e.p./min. No visible cytological changes were produced by this level of radiation. In view of the well-known tolerance of frog eggs to much larger dosage of X-rays, it is fair to assume that radiological effects were negligible in our results.

Radio-autographs

A new variant of conventional methods of making radio-autographs has been devised to fit the purpose of recording radioactivity distribution within a single cell. Our technique consisted essentially of quick-freezing in liquid air, followed by sectioning the cell to a known thickness, and then exposing the section in a light-proof cold box to a photographic plate.

Eggs were rapidly rinsed (5 sec.) in two washes of normal Ringer (7 ml. each), oriented in a 1.8 mm. hole in thin calibrated bronze strips (1.5×3.5 cm. and either 80 or 200 μ thick) and plunged into liquid air. The orientation was such that the axis of the egg was parallel to the surface of the strip. It was calculated that freezing of a 1.7 mm. egg at $-180°$ C. would be completed at the center in about 0.2 sec. This time should be compared with the ten minutes necessary for partial equilibrium of ionic diffusion in the egg at normal temperatures. The bronze strip containing the frozen cell was then placed on a previously cooled brass block (see Fig. 2) and both were transferred to the stage of a dissecting microscope. Using a cooled razor blade mounted in a special non-conducting handle, the frozen egg was sliced down to expose the nucleus. Orientation was

thus re-checked to insure that the plane of section was being made parallel to the egg axis. Improperly oriented eggs were discarded. Subsequent slices through the lateral third of the nucleus were made, making the surface even and parallel with the bronze strip. The nucleus always stood out sharply as a white circle in a grey background of frozen cytoplasm. Finally the strip was inverted and the cell was sectioned similarly from the other side. Frozen sections were conveniently temporarily stored in a cold slotted brass holder in an insulated box, cooled with solid CO_2.

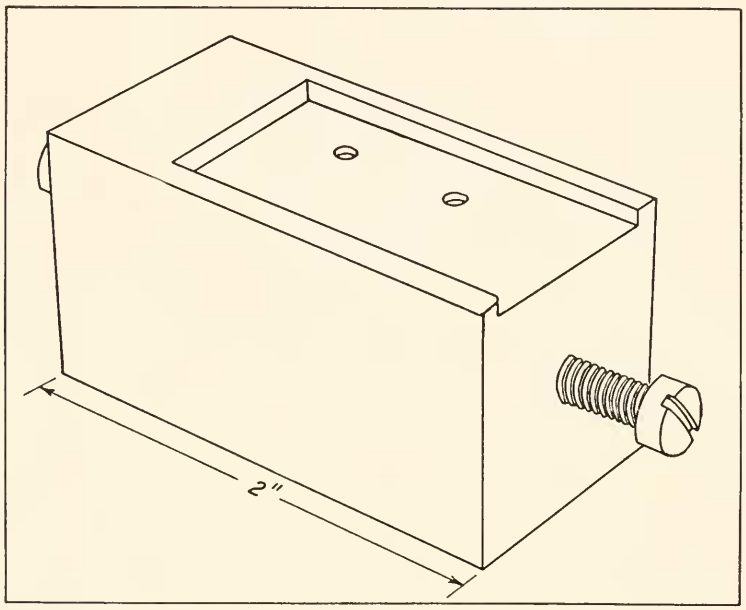

FIGURE 2. Brass cooling block for holding frozen egg and supporting strip during sectioning. Note that both holes corresponding to eggs in holder are displaced laterally from the main axis of the block, so that the strip, when inverted after first sectioning, brings cut surface against flat portion of the block. Screw at each end makes convenient attachment for handle.

Actual exposure of the section to film took place in a well-insulated chest fitted with a copper bottom and copper slide holder. One hundred pounds of dry ice kept the temperature in the slide holder at approximately $-40°$ C. for a week. Medium-contrast lantern slide plates [1] were laid in the slide holder, emulsion side up, and the cold bronze strips with the frozen sections were laid on the plates, being weighted to insure even contact. With the activity of our solutions, it was found necessary to expose from two to twelve hours to provide the desired darkening. Plates were developed in Eastman D-19 developer.

A typical calculation indicates the amounts of radioactivity involved. A section of a frog egg 80 μ thick contained 2.0×10^{-8} g. of exchanged sodium. This sample

[1] We have learned from Dr. Kenneth Endicott, of the National Institutes of Health, that Ansco non-screen X-ray films are particularly useful where only low intensities of radiation are present.

gave 320 counts/min. on the counter. The actual number of disintegrations occurring in the egg section was approximately 20/sec. An exposure of four hours sufficed to give a good darkening showing a clear differential distribution between nucleus and cytoplasm. This amounts to 288,000 electrons, about half of which went into 0.026 cm^2 of the plate emulsion.

Results

Sodium content of ovarian eggs

The sodium content of frog eggs is not well known. Bialaszewicz (1929) has given an approximate figure of 42 mg. Na/100 g. wet weight of eggs of *Rana temporaria.* It seemed desirable, therefore, to determine sodium on our experimental animals. For this purpose, both ovaries were removed from six animals. The ovaries were rinsed quickly in distilled water, blotted, weighed, and cautiously ashed over low heat by concentrated HNO_3, followed by concentrated H_2SO_4.

After removal of a small amount of insoluble material, probably $CaSO_4$, the sodium was determined as $(UO_2)_3ZnNa(CH_3COO)_9 \cdot 6H_2O$ according to the method of Barber and Kolthoff (as given in Hillebrand and Lundell, 1929). Results obtained from six pairs of ovaries gave a preliminary value of 137 mg. Na per 100 g. wet weight of eggs (Duryee and Abelson, 1947). Further study has shown these results to be too high and that PO_4 interferes with the accuracy of the method. This is at least in part due to the formation of an insoluble uranium-phosphorus compound which tends toward giving high results for the sodium value. Accordingly the procedure was modified. Following wet ashing, excess acid was driven off, the residue dissolved in distilled water, and the solution made alkaline with NH_4OH. A 25 per cent barium acetate solution was added until no further precipitate formed, followed by a few drops excess. The mixture was allowed to stand for half an hour and then centrifuged. The precipitate was washed twice with distilled water and the washings added to the first supernatant. The solution was evaporated to dryness, the residue dissolved in 1 ml. of distilled water, and the sodium precipitated with the uranyl zinc acetate reagent. The following results were obtained from six pairs of ovaries:

Wt. of ovaries (grams)	mg. Na	mg. Na/g. ovary
8.1	6.8	0.84
9.3	7.40	0.80
9.0	7.4	0.82
8.5	7.0	0.82
7.0	5.9	0.80
8.4	7.1	0.84
	Average	0.82 mg. Na

There remained the question of how well the sodium content of the whole ovary approximates that of the eggs. Since the connective tissue, blood vessels and peritoneum formed only a minor fraction of the ovary, it seemed that the error introduced by neglecting this factor would be small. A sodium determination on separated eggs gave 0.80 mg. Na/g. wet weight. Eggs averaged 1.76 mm. in diameter with a volume of 2.86 mm^3, a density of 1.10, and a calculated sodium content of 25.8×10^{-7} g. Na per egg.

Water content of eggs

In analyzing the results, it was found desirable to measure the water content of the eggs. Accordingly four determinations were made—two with separated eggs and two with ovaries. Excess moisture was blotted up with filter paper. The eggs were weighed and then dried to constant weight at 120° C. for one hour. A water content of 52 per cent was found. This corresponded to 1.63 mg. of water for our standard 1.76 mm. diameter egg.

An interesting observation gave evidence on the water content of the nucleus. In six sets of eggs which were frozen whole, the half sections, when thawed and dried, showed a negative mold of the nucleus with only a flake remaining. This indicates the relatively higher water content of the nucleus above that of the yolk-cytoplasm.

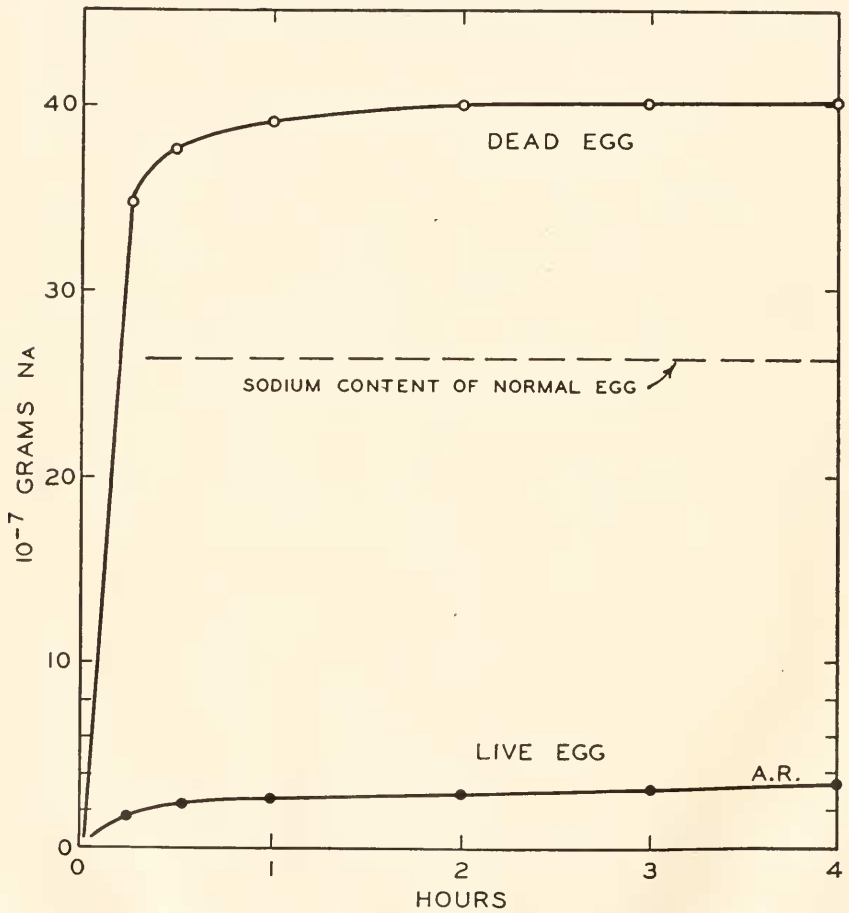

FIGURE 3. Comparison of uptake of Na^{24} by live and dead eggs in amphibian Ringer. Average value of sodium content of single egg, as determined by chemical analysis, shown by dotted line. Each point represents average measurements on ten separate cells.

Sodium exchange

In the course of the research a total of twenty experiments were performed involving 400 individual eggs. The experiments gave a reproducible set of values with a probable variation of plus or minus ten per cent. A comparison of the exchange of sodium in live and dead eggs is shown in Figure 3. Each point on the curve represents an average of ten measurements. In the case of live eggs, the following features are to be noted: Initially there was a rapid penetration of the traced sodium into the egg. Within an hour, however, the amount of the traced substance within the cell ceased to increase. The level reached amounts to 12 per cent of the total sodium content of the egg. By testing samples of cytoplasm and later by making radio-autographs, it was shown that the traced sodium was principally inside the egg.

With dead eggs (Fig. 3) the exchange of sodium was extremely rapid. The amount of traced sodium which could be found in the egg rose above the total sodium content of the live cell as determined chemically. This entrance of extra sodium can be related to at least two facts: We have observed a loss of potassium from the cell on death. The second fact is that the sodium content of amphibian Ringer for a volume equal to that of the frog egg is 78.4×10^{-7} g., or more significantly, the sodium present in a volume of Ringer equal to that of the cell's water content is 42.4×10^{-7} g. Na.

To study further the nature of the sodium uptake process, eggs were first exposed to radioactive Ringer, washed and then immersed in an inactive Ringer solution. The results are shown in Figure 4. During the first hour, a typical curve was obtained for penetration of the traced sodium. The downward curve was obtained during exposure to inactive Ringer. The time required for the traced sodium to leave the cell was about the same as the time involved in the initial penetration. Chemical analysis showed that the total sodium content of the egg did not change during these experiments. When dead eggs containing tracer sodium are exposed to inactive Ringer, the traced sodium likewise leaves the egg very quickly.

Since calcium is thought to be important in permeability relationships, experiments were designed to investigate the effect of this ion on sodium exchange. Three solutions were made up with tracer sodium: The first, an ordinary Ringer solution; the second, with calcium not present; and a third, with double the usual calcium content. When eggs were exposed to these solutions and measured as before, the curves shown in Figure 5 were obtained. These curves show that calcium only slightly influenced that fraction of the sodium (12 per cent) which is exchangeable. After several hours, however, lack of calcium resulted in an increased exchange of the sodium.

A series of experiments was made to determine the effect of temperature on exchange of sodium. Experiments were conducted at 9° C., 20° C., and 30° C. While the speed of penetration of sodium was somewhat faster at the higher temperature, the level reached after an hour was almost identical in all cases. After three hours approximately 6×10^{-7} g. Na was found per cell, while the low temperature values were essentially those of the controls at 20° C. It is apparent, therefore, that sodium exchange is not highly dependent on cellular metabolism.

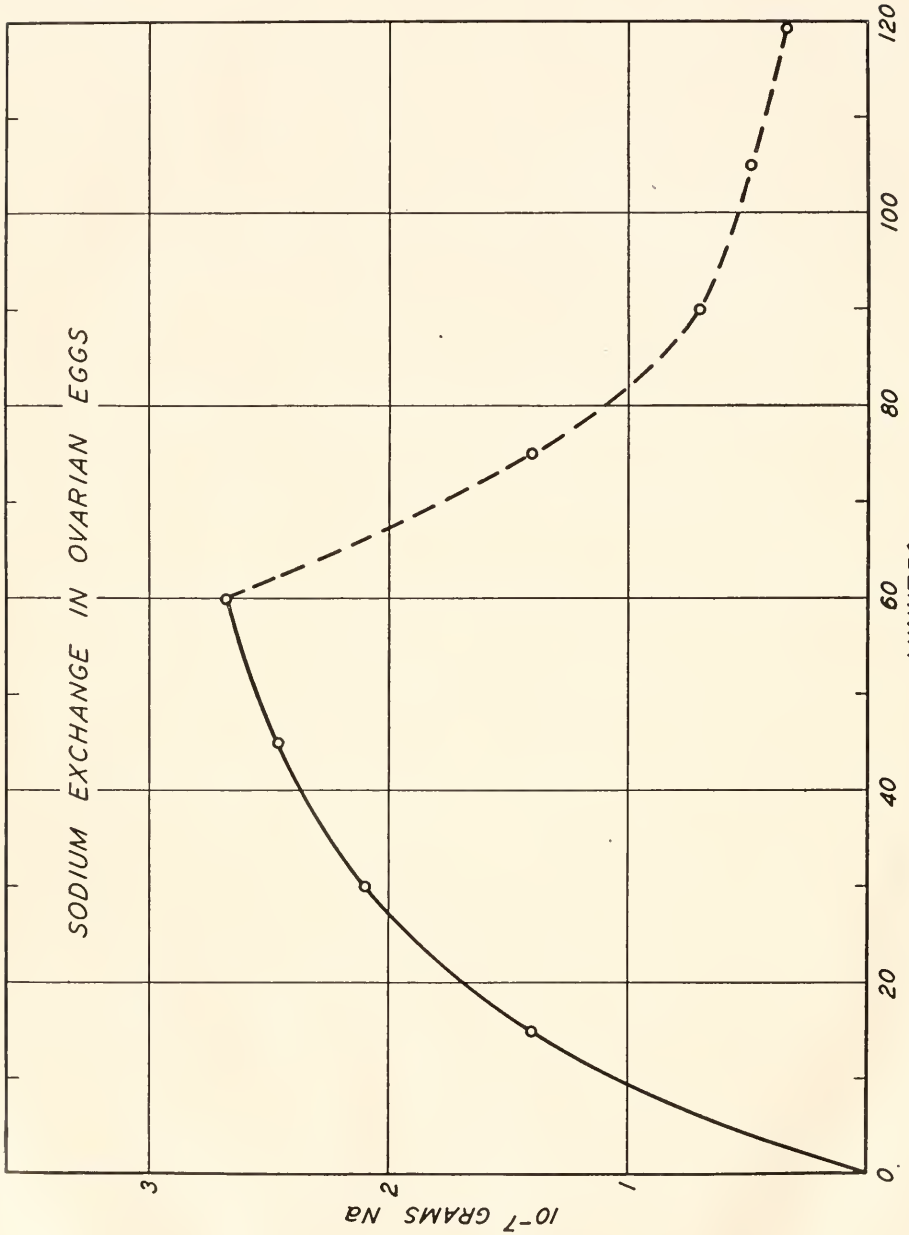

FIGURE 4. Comparison of rate of uptake of Na^{24} in a radioactive Ringer solution with the rate of "washing-out" in a normal Ringer medium.

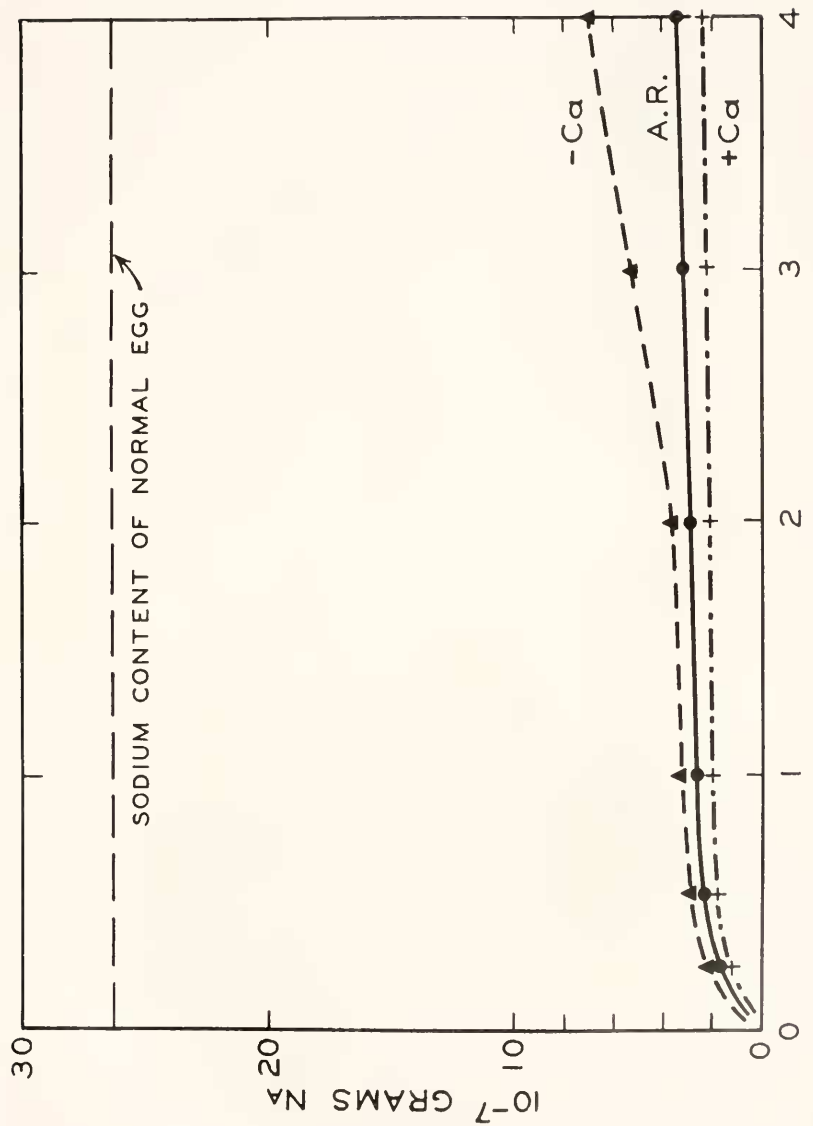

FIGURE 5. The influence of calcium on the rates of sodium exchange in single cells. Calcium-free Ringer (−Ca) ---; normal amphibian Ringer (Ar) solid line; Ringer with double calcium content (+Ca) -·-·-.

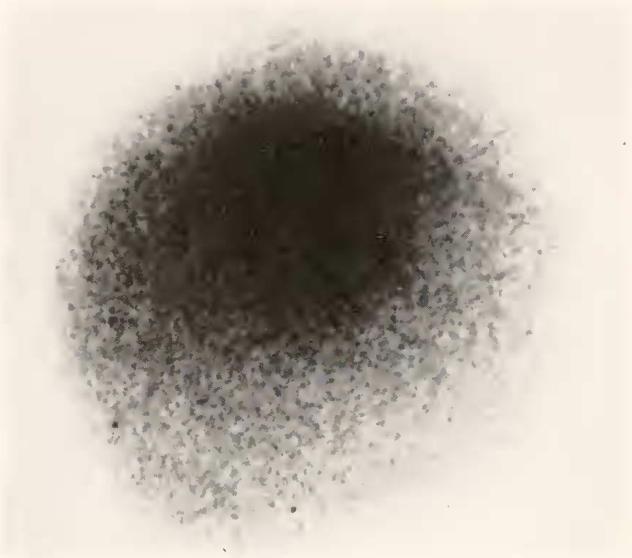

FIGURE 6. Radio-autograph of ovarian egg exposed to Na^{24} for thirty minutes. Cell was frozen in liquid air and sectioned parallel to main axis. Dark portion in upper third corresponds to size and position of the cell nucleus.

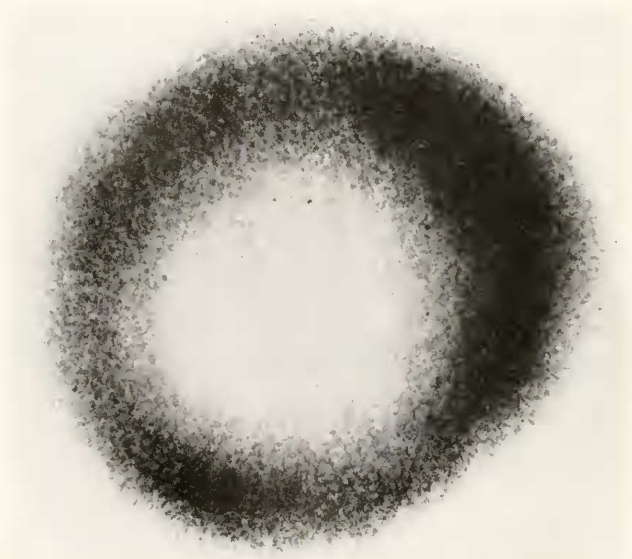

FIGURE 7. Radio-autograph of dead ovarian egg exposed to Na^{24} for five minutes. Width of "ring" is proportional to diffusion rate of sodium inside the cell.

The question of where the sodium goes inside a cell seemed of fundamental importance. Many attempts were made to answer this question with but little success. It was of course possible to isolate single nuclei from exposed eggs. Such nuclei were washed twice in calcium-free nuclear medium to remove outside "cytoplasmic" sodium and were then placed individually on plastic cups for measurement with the Geiger counter. The counts per nucleus were always significantly higher than background, but showed disappointing variation. .It was concluded that the washing process introduced an uncontrollable variable. Consequently, we turned to the radio-autograph technique. In Figure 6 is shown an enlargement of a typical radio-autograph of a frog egg frozen in liquid air and sectioned meridianally. Of 47 autographs of single cells exposed to Na^{24} for more than 30 minutes, all showed denser silver particles in the nuclear area than in the cytoplasm, when the plane of section was through the germinal vesicle. In those autographs of eggs sectioned to one side of the nucleus, a uniform density was found throughout the endoplasmic area.

In the analysis of sodium diffusion rates inside the cell, advantage was taken of the radio-autographic records of penetration. In Figure 7 is shown an enlargement of a radio-autograph obtained by exposure of a dead egg to radioactive Ringer for five minutes followed by washing, freezing, and sectioning. When live eggs were exposed to the active Ringer, for five minutes, followed by standard technique, a similar ring-shaped autograph was also obtained with the density of the darkening much less.

Discussion

To serve as a basis of comparison, a calculation [2] has been made of the time required for diffusion processes to exchange sodium in an element of fluid the size

[2] An exact calculation of diffusion in an object similar to the frog egg would be practically impossible. First, there is the inhomogeneity of nucleus and cytoplasm. Second, is the fact that only a part of the sodium in the egg exchanges. One important factor in the calculation is that these experiments trace the behavior of Na^{24} in a medium under conditions where there is no net transport of sodium or chloride ions. Therefore, in calculating the diffusion of Na^{24} in Ringer solution, one should use the self-diffusion coefficient of Na^{+} rather than that of NaCl. Fortunately, the self-diffusion of Na has been measured in sodium chloride solutions by Jehle (1938) and in sodium iodide solutions by Adamson (1947). In the experiments the concentration of salt is the same on both sides of a diaphragm, and a very small amount of radioactive sodium is added to one of the solutions. From the rate of appearance of tracer sodium in the second solution the self-diffusion coefficient can be measured. The value observed was 1.2×10^{-5} cm^2./sec. in 0.113 M NaCl solution, and 1.23×10^{-5} cm^2./sec. in 0.113 M sodium iodide solutions.

A calculation giving useful information regarding diffusion into a sphere can be made by employing formulas presented by Barrer (1941). The formulas have been applied to "Ionic Exchange Absorption Processes" by G. E. Boyd et al. (1947). In the case of a completely permeable sphere initially free of the diffusion ion, the time required for the solute to reach half of its equilibrium value is given by

$$Q/Q_\infty = 1/2 = 1 - 6/\pi^2 \sum_{n=1}^{\infty} 1/n^2 \exp.\ (-D\pi^2 n^2 t/r^2)$$

where Q_∞ is the equilibrium value of the quantity of traced sodium, Q is the value at any time t, D is the diffusion coefficient of Na taken here as 1.23×10^{-5} cm^2./sec., and r the radius is 0.088 cm. By a method of successive approximations the above equation is satisfied when $D\pi^2 t/r^2 = 0.3$ and $t = 19$ seconds.

of a frog egg. Assuming no retardation at the surface of the object and a diffusion coefficient of 1.23×10^{-5} cm^2/sec. internally, the time required for half of the sodium to exchange is 19 seconds. In the case of the actual egg, the time required for half of the exchangeable sodium to exchange is 15 minutes. The exchange of sodium in the egg may be slower for at least two reasons: delay in passing through the cell membrane, and the existence of a low diffusion coefficient within the egg. The fact that we have obtained "ring" radio-autographs after exposure of five minutes is explainable only on the basis that the movement of sodium in the egg is much slower than in Ringer solution, and that the membrane has little limiting effect on the exchange.

On the assumption that the membrane presents no barrier, one can calculate a value for the diffusion coefficient within the egg. Thus, for $Q/Q^{\infty} = 1/2$, $D\pi^2 t/r^2 = 0.3$, and $D = 2.6 \times 10^{-7}$ cm^2./sec.

From the curves given in Figures 3 and 5, it is clear that only 12 per cent of the sodium in the ovarian frog egg is readily available for exchange. Part of the remaining sodium can be exchanged over a period of many hours. Since the factors which govern this slow exchange are wholly within the egg itself, some form of internal blocking must occur. Speculation concerning the mechanism is still unwarranted. We believe that the finding that 12 per cent of the sodium behaves differently from the remainder is of considerable importance to others who are performing tracer experiments. In this experiment, the behavior of the traced substance was not characteristic of the behavior of all the sodium of the cell. Upon the other hand, it would appear that the difficulty raised might be turned into an advantage. One has a technique for studying the various degrees of binding and the chemical activity of cellular components.

It is of special interest that sodium could be traced into the germinal vesicles. Our experiments thus show that the nuclear membrane is permeable to sodium. They also show that sodium is normally present in a cell nucleus. It is especially significant that the concentration of this cation in the nucleus is at least double that of the cytoplasm. The relatively large amount of active sodium found in the nucleus may be connected with the fact that the nucleus has a much higher percentage of water than does the yolk cytoplasm.

Our data on the increased permeability of dead cells to Na^{24} offer a basis for explaining some of the empirical observations of Quimby (1947). Working with second and third degree burns she was able to show that the more severely damaged tissues took up much greater amounts of tagged sodium. These results must be due to the presence of dead cells. Furthermore, she found that addition of hypertonic saline materially aided recovery. It is clear, therefore, that in both sets of observations a fundamental factor is operating. This epitomizes, in the case of sodium, what many physiologists have long believed to be a basic diagnostic character of life—namely, that a living cell can discriminate between ions both quantitatively and qualitatively in its extracellular environment and can build specific internal gradients and unequal distributions, which a dead cell certainly cannot do. As we have shown in the case of the amphibian egg, permeability to radioactive sodium can be used as an indication of life or death in a cell.

Summary

1. The ovarian egg of the frog *Rana pipiens* is freely permeable to Na^{24}.

2. At least two different types of binding limit the internal diffusibility of sodium within the egg. Only 12 per cent of the normal sodium is readily exchangeable. The remainder exchanges very slowly.

3. The implications of finding non-equilibration of such a simple ion as sodium are presented.

4. A new technique for making radio-autographs of single cells shows that after half an hour sodium is distributed almost uniformly throughout the cytoplasm.

5. Calculations based on rate of exchange of sodium into the egg plus radio-autograph evidence give a value of 2.6×10^{-7} cm^2./sec. for the diffusion coefficient of sodium within the egg.

6. At equilibrium the nucleus possesses approximately twice as much traced sodium per unit volume as the cytoplasm.

7. By direct chemical analysis the sodium content of frog eggs was found to be 0.082 per cent of the wet weight.

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